Supplementary Information for

Females become infertile as the stored sperm's oxygen radicals increase

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Supplementary Methods

Bedbug culture. Bedbugs were cultured at 25 °C and 70% r.H. as described previously (for the origin of stocks, further culture details and life-history characteristics[41,44,45]). Virgin males and females were obtained by separating nymphs from the mass cultures and keeping them in isolated tubes, equipped with filter paper. Males and females aged 14 days post-eclosion were used in this experiment, unless otherwise stated. We used same-aged males to reduce the variation in sperm stem cell ageing related to male age[15,47]. All males were from the same population and raised under identical condition in order to reduce between-male variation in sperm quality decline. Females were taken from the same population as males to reduce variation arising from possible, non-coevolved female-sperm interactions.

Mating protocol. A single male and a single female were introduced into a small petri dish (diameter 5 cm). After the male introduced his genitalia the pair was left undisturbed for a prescribed amount of time, upon which they were separated using soft forceps[48]. After the prescribed number of matings females were kept individually and fed according to a predetermined protocol (see below).

Separating sperm number from sperm quality effects. Previous approaches to study the effect of declining quality of stored sperm included i) to compare the onset of infertility between singly and repeatedly mated females or between briefly and normally mated females[56-62], ii) using female lines of low or high early fecundity[63] or iii) comparing reproductive parameters within individual females over the duration of sperm storage[6,16,57-59,61,62].

Interactions between sperm quality and quantity also exist, including increased sperm per egg-use when sperm quality decreases[6,60], storing fewer sperm of higher age or lower quality[65], a negative correlation between sperm density and sperm mortality[14,66,67] or the fact that large ejaculates contain a larger total number of young sperm[14]. However, if sperm quantity affects sperm quality, the predicted fitness effects would be the same as those observed for sperm number manipulation, none of which were found here.

Recovery of female fertility. We studied whether female fertility can recover spontaneously or only in response to mating. Forty-six females, 1-3 weeks old and weekly fed, were individually mated for 60 seconds to virgin, 1-3 weeks old, weekly fed males. Nineteen of these females were remated on the day infertility was recorded. The occurrence of fertile eggs subsequently laid by these females would establish that sperm, rather than female senescence, is the cause of infertility in our experiment. The remaining twenty-seven females were not remated but used to assess whether female fertility can spontaneously recover. The presence of live sperm in ten of these females was examined two weeks after the onset of infertility, when females laid clutches with only infertile eggs (see below, *The presence of live sperm at infertility*).

The presence of live sperm at infertility. Run in parallel to the recovery assay explained above, an additional 18 females, aged 1-3 weeks and fed weekly, were mated once for 60 s. They were examined for the presence of live sperm in the storage organ on the day they were scored infertile. We also examined the ten females who laid all-infertile clutches two weeks after the start of infertility (see above).

In bedbugs the sperm cells in the female storage organ are tightly associated with female cells (Fig. 1B). In a pilot study, dissection of the female storage organ was not always achieved. If it was, individualization and homogenization of sperm cells for counting proved difficult and so was not employed. Instead, the female sperm storage organ was placed in a 2 µL droplet of Grace's Insect Medium with the same amount of SPERM DEAD/LIVE solution according to the manufacturers' instruction. The sample was observed at x400 magnification in an epifluorescent microscope with UV excitation. The proportion of dead sperm (fluorescing red) and live sperm (fluorescing green) was estimated (Fig. 1A).

Dissection was successful in 11 out of 18 allocated females and in 7 out of the ten females that laid all-infertile clutches two weeks after the onset of infertility (overall 64%). In 10 out of the 11 successfully dissected females, a mean of 85.6% (range 50-100%) of the sperm were alive when infertility started. Six out of 10 all-infertile females had still stored live sperm (50-100%).

Five dissections failed completely and in five of the unsuccessful dissections (4 allocated, 1 all-infertile) no or no live sperm were detected. The absence of live sperm can be attributed to sperm damage during dissection or the cytotoxic nature of the staining chemicals. As a consequence, the presence of dead sperm includes sperm that were dead at infertility but also false negatives (e.g. sperm that had died during dissection). However, the presence of live sperm in 16 females is positive proof that at infertility sperm stored by the females were still alive.

Time-resolved microfluorimetry. We recorded the fluorescence decay of sperm cells loaded with PBA[32]: A laser NL100 (SRS) delivered monochromatic pulses at a wavelength of 337 nm with a half amplitude pulse-width of 3 nanoseconds. The excitation beam is concentrated to an approximate diameter < 40 µm (Fig. 1A) onto the microscopic sample by means of an objective (40×, Unitron). Emitted photons were collected and focused on a photomultiplier (RCA 1P28). The signal was digitized using a digital oscilloscope (Tektronix TDS 3032C). A 404 nm bandpass filter (half bandwith 40 nm) was placed into the emission path to select the pyrene emission.

The fluorescence decay was partitioned into three exponentials and their lifetime and amplitude obtained as described previously[32,34]. Briefly, the shortest two decays τ_1 and τ_2 correspond to the autofluorescence of the free and protein-bound form of NAD(P)H (see above). In order to relate their amplitudes a_1 and a_2 directly to changes in the sperm metabolic rate, we calculated the relative change in metabolic rate as $[a_2/(a_1 + a_2)]/[(a_{2reference}/(a_{1reference} + a_{2reference})]$ where $a_{1reference}$ and $a_{2reference}$ are the mean fluorescence amplitudes of freshly ejaculated sperm for free NAD(P)H (a_1) and bound-NAD(P)H (a_2). The third time constant ($\tau_3 > 100$ nanoseconds) is characteristic of pyrene derivatives.

Absolute radical concentrations cannot be obtained with our method but the change in fluorescence lifetime is proportional to a change in ROS production and can be calculated applying the Stern-Volmer equation[28] to the fluorescence lifetime data, τ_3:

$$[ROS]_{sample} = [\tau_{reference} (\tau_0 - \tau_{sample})] / [\tau_{sample} (\tau_0 - \tau_{reference})] \times [ROS]_{reference}$$

where τ_{sample} was the fluorescence lifetime measured in the sample, $\tau_{reference}$ the mean fluorescence lifetime of all samples of ejaculated sperm, and τ_0 (165 nanoseconds) the fluorescence lifetime measured in the absence of ROS production (cells dead, fixed with Baker solution). The mean of all samples of ejaculated sperm was set at 100%, so that plots give ejaculate-specific values relative to that mean.

Statistical analysis

Time-failure analysis. Time-failure analysis was employed to detect differences between females in the onset of infertility. Using the statistical package "survival" in the program R 2.15.1.[68]. The fit of six age-dependent hazard models[68] was compared using the *anova* procedure. The model "extreme value"[69] provided the best fit and was used. Model reduction procedures were only applied if reduced models provided a better fit than the full model, as tested by *anova* procedure[69].

Generalized linear models. The effect of storage, storage duration, handling time, and their interactions on fluorescence lifetime and metabolic rate were tested using the lme4 package in the program R 2.15.1.[69]. Because the sperm of some males was analyzed in both the male and the female we used generalized linear mixed effects models (*glmer*) with sample ID as the random factor. Non-significant interactions were removed from full models but only excluded if the reduced model provided a better fit than the full model, as tested by *anova* procedure[69].

Chemicals

1-pyrenebutyric acid (PBA) (Acros organics, Geel, Belgium) was dissolved in ethanol (95%) to 50 µM and stored at 4°C. Staining was carried out with 1 µM PBA (2% ethanol). Dulbecco's Phosphate-buffer saline (DPBS) (Cambrex, Verviers, Belgium).

Supplementary References

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Supplementary Table S1. Time-failure analysis of the onset of infertility in female bedbugs, *Cimex lectularius* (N = 54) affected by sperm numbers transferred, and sperm numbers used. The occurrence of the second infertile egg was used as an indicator of beginning infertility (A). The results did not change if the first infertile egg was used (B). Copulation duration was used as a proxy for sperm numbers transferred, weekly alternating feeds, as opposed to weekly feeds reduced the number of egg-laying events and so was used as an indicator of the numbers of fertilization events, or sperm used. Note that although sperm numbers used approached significance, it was in the opposite direction of what was predicted.

A) Second infertile egg as proxy of onset of infertility

Model: Onset of infertility ~ sperm number transferred * Sperm numbers used, distribution = "extreme".

	Value	s.e.	z	p
(Intercept)	7.2471	1.748	4.1462	3.38e-05
Sperm number transferred	0.1226	0.384	0.3194	0.749
Sperm numbers used	2.0877	1.118	1.8671	0.062
Interaction	0.0214	0.246	0.0869	0.931

B) First infertile egg as proxy of onset of infertility

Model: Onset of infertility ~ sperm number transferred * Sperm numbers used,

distribution = "extreme".

	Value	s.e.	z	p
(Intercept)	9.163	2.153	4.256	2.08e-05
Sperm number transferred	-0.102	0.474	-0.214	0.830
Sperm numbers used	-0.481	1.314	-0.366	0.714
Interaction	0.252	0.287	0.879	0.380

Supplementary Table S2. Reduced model for the effect of storage duration on sperm oxygen radicals production. Fluorescence lifetime of an oxygen radicals-sensitive probe (PBA) in sperm stored for ten weeks (long-term storage) are compared to short-term (6 h) and intermediate term (3.5 wks) storage and in relation to handling time. Interactions terms in the full model were not significant and removed. Note that positive effects, i.e. increased estimates, signify reduced oxygen radicals production.

Model:
glmer(fluorescence lifetime ~ handling+ storage time (short or intermediate) +
(1|sample.ID)
Random effects:

Groups	Name	Variance	s.d.
sample.ID	(Intercept)	7.527	2.743
Residual		10.190	3.192

	Estimate	s.e.	t	P
(Intercept)	150.053	1.551	96.74	< 0.0001
handling	-0.122	0.041	-2.96	0.0039
intermediate	5.680	1.544	3.68	0.015
short-term	3.390	1.710	1.98	0.061

Supplementary Table S3. Metabolic rate of stored sperm for ten weeks compared to short-term (6h) and intermediate-term (3.5 wks) storage and in relation to handling time. Although there was an interaction effect of handling time and intermediate sperm storage, the metabolic rate at intermediate sperm storage intervals was significantly lower than long-term stored sperm.

glmer(metabolic rate ~ handling time * storage time (short or intermediate) + (1|sample.ID)

Random effects:

Groups	Name	Variance	s.d.
sample.ID	(Intercept)	0.0038	0.0614
Residual		0.0052	0.0723

	Estimate	s.e.	t value	P
(Intercept)	0.4429	0.056	7.921	< 0.0001
handling	-0.0011	0.002	-0.543	0.703
intermediate	-0.1822	0.070	-2.600	0.009
short-term	0.0497	0.070	0.714	0.242
handling:intermediate	0.0058	0.003	2.264	0.017
handling:short-term	-0.0009	0.003	-0.360	0.361

Supplementary Table S4. The effect of sperm metabolic rate, sex, sample handling time and their interactions on the oxygen radicals production of sperm of the bedbug *Cimex lectularius*. None of the interactions were significant and stepwise removed from the full model, based on t-values. Note that positive effects, i.e. increased estimates, signify reduced oxygen radicals production. The sperm of some males could be measured in both the male and in the female, therefore sex was nested in sample ID.

Model used:

glmer(Fluorescence lifetime ~ Metabolic rate + sex + handling + (1|sample.ID/sex)

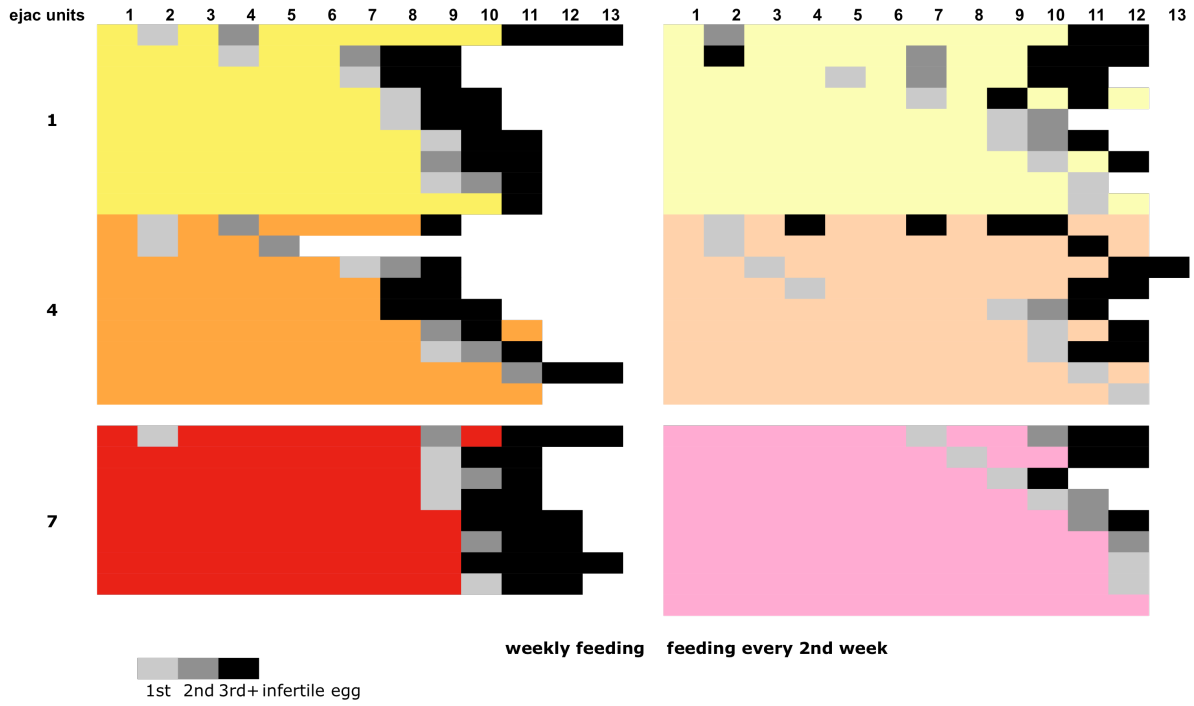
Random effects:

Groups	Name	Variance	s.d.
sex:sample.ID	(Intercept)	8.7511e+00	2.9582e+00
sample.ID	(Intercept)	4.1291e-11	6.4258e-06
Residual		8.0671e+00	2.8403e+00

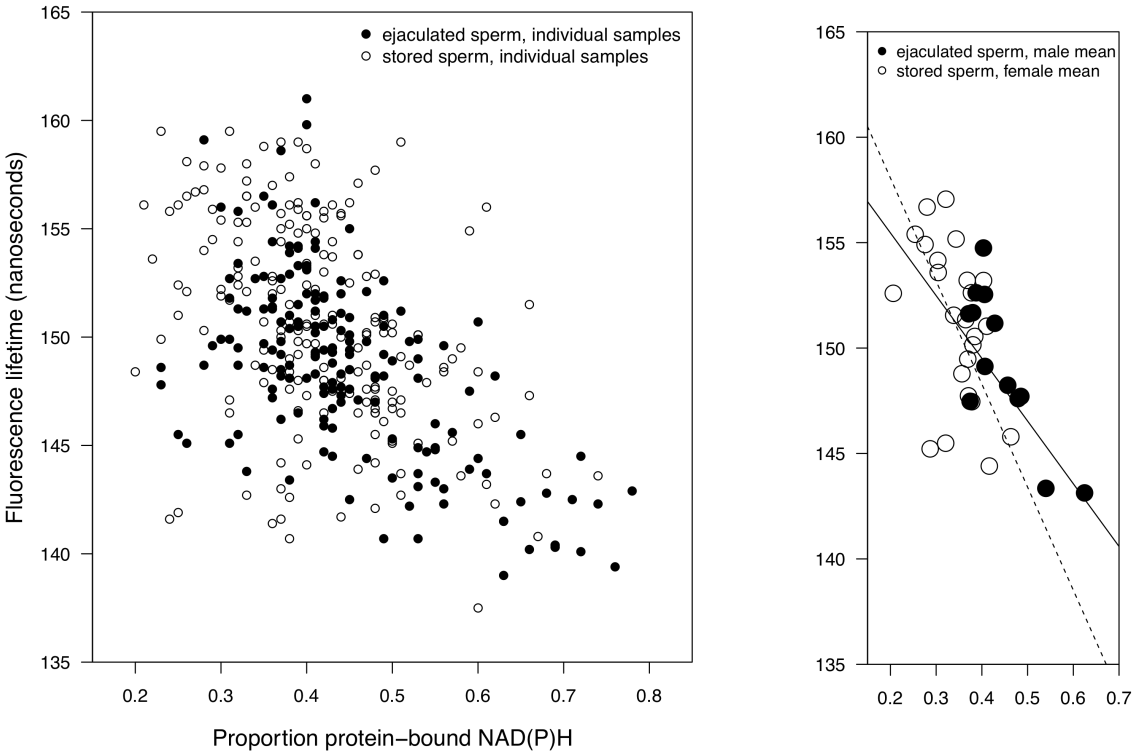
Fixed effects:

	Estimate	s.e.	t value
(Intercept)	158.95	1.144	138.90
Metabolic rate	-13.30	1.934	-6.87
Male	-1.216	1.103	-1.10
Handling time	-0.121	0.027	-4.42

Supplementary Figure S1. Female fertility in relation to sperm numbers. The onset of infertility in 54 sexually isolated female bedbugs in relation to the numbers of sperm transferred (1 ejaculate unit: yellow, 4 ejaculate units: orange, 7 ejaculate units: red) and the numbers of sperm used (weekly egg laying: strong colours or biweekly egg-laying: shaded colours) over 12 to 13 weeks. Each row represents an individual female and its first (light grey), second (dark grey) and third (black) infertile egg. No symbols are drawn when females stopped laying.



Supplementary Figure S2. The relationship between the proportion of protein-bound NAD(P)H (metabolic rate) and the fluorescence lifetime of the oxygen-sensitive probe PBA (inverse of oxygen radicals production rate) in sperm of the bedbug *Cimex lectularius*. Sperm were either extracted from the male (filled circles, N = 13) or from the female (empty circles, N = 20) sperm storage organs at various time points after mating. Some females were sampled at two time points. Therefore, sample sizes differ from those given in Fig. 1.



Supplementary Figure S3. The decrease in sperm volume across three or four successive matings in male bedbugs *Cimex lectularius*. The decrease was measured as the repeated decline of the volume of the sperm storage organ of individual male bedbugs (white vesicles in inset picture).